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ORIGINAL ARTICLE

Changes in the expression of substance P in nerve fibres of the colonic mucosa in dogs suffering from inflammatory bowel disease

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ABSTRACT

Due to its difficult diagnosis and complicated treatment, inflammatory bowel disease (IBD) in dogs is a challenge for the veterinarian. Several aspects connected with pathological changes during IBD still remain unknown. Since one of these aspects is the participation of intestinal innervation in the evolution of the disease, the aim of this study was to demonstrate changes in the number and distribution of intramucosal colonic nerve fibres immunoreactive to substance P (SP) arising as the disease progresses. SP is one of the most important neuronal factors in intestinal innervation which, among other tasks, takes part in the conduction of pain stimuli. Using routine immunofluorescence technique, the density of nerve fibres containing SP was evaluated within mucosal biopsy specimens collected from the descending colon of healthy dogs and animals suffering from IBD of varying severity. The results of the study indicate that during severe IBD the number of nerve fibres containing SP located in the colonic mucosal layer increases in comparison to control animals. The number of SP-positive intramucosal nerves amounted to 10.99 ± 2.11 nerves per observation field in healthy dogs, 14.62 ± 2.86 in dogs with mild IBD, 14.80 ± 0.91 in dogs with moderate IBD and 19.03 ± 6.11 in animals with severe IBD. The observed changes were directly proportional to the intensity of the disease process. These observations may suggest a role of this neuronal substance in pathological processes occurring during IBD. Although the exact mechanism of the observed changes has not been completely explained, the results obtained in this investigation may contribute to improving the diagnosis and treatment of this disease, as well as the staging of canine IBD in veterinary practice.

KEYWORDS

colon, dogs, innervation, inflammatory bowel disease, substance P

INTRODUCTION

Canine inflammatory bowel disease (IBD) is a set of enteropathies of idiopathic and inflammatory origin, characterised by persistent or recurring gastric signs (Jergens et al., 2003). It should be noted that the aetiology of IBD has not yet been completely explained (Simpson and Jergens, 2011). The most important factors taking part in the emergence of the disease are genetic and environmental factors, microbiota, food antigens, as well as inadequate host responses to selected drugs (Bathia and Tandon, 2005; Allenspach et al., 2007; Suchodolski et al., 2010; Simpson and Jergens, 2011; Sheehan et al., 2015). The clinical signs of IBD in dogs are non-specific and include vomiting, diarrhoea, weight loss, bloating, abdominal cramping and loss of appetite (Jergens et al., 1992). The above-mentioned signs are

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accompanied by histopathological changes in the intestinal wall. In recent times, attention has been focused on the participation of intestinal innervation in pathological processes connected with IBD (Gonkowski et al., 2013; Rychlik et al., 2017; Brinkman et al., 2019), but the roles of particular neuronal active substances in the development of this disease are still not clear.

One of these neuronal factors is substance P (SP) – a peptide which, together with neurokinin A and B and neuropeptide K and γ, belongs to the tachykinin neuropeptide family (Maggi, 2000; Shimizu et al., 2008). Although SP was described for the first time even before World War II, its functions within the gastrointestinal (GI) tract have not been completely explained. Previous investigations have described SP both in the enteric nervous system located in the wall of the GI tract and in extrinsic innervation of the intestine in a wide range of mammalian species, including humans (Deguchi et al., 2001; Shimizu et al., 2008; Gonkowski, 2013; Rytel and Calka, 2016). It is also known that this substance may act via three types of G protein-coupled receptors belonging to the NK receptors (NK1, NK2, NK3), all of which have been also observed in the intestinal nervous structures (Maggi et al., 1993). SP within the GI tract may play multiple roles. In particular, it influences the contraction of intestinal muscles and the secretory activity of the GI tract, regulates the blood flow in the mesenteric and intestinal vessels and participates in the conduction of sensory and pain stimuli and epithelial ion transport (Brunsson et al., 1995; Brehmer et al., 2004; De Fontgalland et al., 2008; Shimizu et al., 2008). Moreover, SP affects immunological processes via NK1 receptors located on lymphocytes and macrophages (Mantyh et al., 1994; Koon and Pothoulakis, 2006; Gross and Pothoulakis, 2007). It should be noted that the functions of SP differ depending on which type of receptor is stimulated. For example, stimulation of the NK3 receptor causes the contraction of the intestinal muscles, while stimulation of the NK1 receptor results in relaxatory effects (Shimizu et al., 2008). It is also known that the number and distribution of intestinal nervous structures containing SP may undergo changes under a wide range of pathological processes (Vasina et al., 2006; Gonkowski, 2013; Rytel and Calka, 2016). These changes are probably connected with the participation of SP in inflammatory processes. Previous studies have described this substance as a potent proinflammatory factor influencing the secretion of cytokines, including interleukin-1 and tumour necrosis factor alpha (O'Connor et al., 2004; Koon and Pothoulakis, 2006; Gross and Pothoulakis, 2007).

Although some studies have reported that SP may participate in pathological processes connected with IBD in humans (Bernstein et al., 1993; Holzer, 1998; Taylor and Keely, 2007), the knowledge concerning this issue is relatively scanty, and the influence of IBD on the distribution of intestinal nervous structures containing SP in the canine GI tract has not been studied at all. On the other hand, based on previous studies, an increase in SP levels may contribute to intestinal changes specific for IBD (Gross and Pothoulakis, 2007; Sideri et al., 2015), but these mechanisms are also not fully understood in dogs. Therefore, the objective of the present study was to compare of the number of substance P-like immunoreactive (SP-LI) nerve fibres in the colonic mucosal layer in healthy dogs and in dogs with different IBD severity scores and to verify if SP, as a neuronal factor in nerves supplying the descending colon, takes part in mechanisms connected with IBD. The results will broaden the knowledge about the role of SP in pathological processes associated with canine IBD and could contribute to improving the diagnosis and treatment of this disease, as well as the diagnostic evaluation of the stage of canine IBD in veterinary practice.

MATERIALS AND METHODS

This study included 28 crossbred dogs of both sexes, 15 males and 13 females, with body weights ranging from 15 to 25 kg and an age range from 6 to 10 years. All activities connected with the experiment were performed according to the permit issued by the Local Ethics Committee for Animal Experimentation in Olsztyn (Decision No. 47/2009/DTN). Dogs suffering from IBD were patients of the Veterinary Clinic of the University of Warmia and Mazury in Olsztyn. The patients were subjected to a comprehensive diagnostic procedure including clinical, laboratory, endoscopic and histopathological examinations of the intestinal mucosal layer. Systemic diseases, antibiotic-responsive enteropathy, food-responsive enteropathy and parasitic infestations were excluded in all patients according to the recommendations of the World Small Animal Veterinary Association Gastrointestinal Standardization Group. Seven healthy dogs, screened with the same tests as the experimental group, constituted the control group. Animals with confirmed IBD were divided into three groups of 7 dogs each based on the canine IBD activity index (CIBDAI) and histopathological scores to define the severity of pathological processes (Jergens et al., 2003):

- Group I – mild IBD, CIBDAI score: 4–5 points, histopathological score: '+'
- Group II – moderate IBD, CIBDAI score: 6–8 points, histopathological score: '++'
- Group III – severe IBD, CIBDAI score: 10–16 points, histopathological score: '+++'

Interestingly, in spite of the fact that during canine IBD complete agreement between the clinical picture of the disease (CIBDAI) and the histopathological scores does not always occur, in dogs included in the study the above-mentioned correlation was accidentally noted, although full agreement of CIBDAI and histopathological scores was not a criterion of inclusion in the study.

Fragments of the mucosal layer examined in this study were obtained during colonoscopy using an FB-50U-1 biopsy forceps with a diameter of 3.7 mm (Olympus). Four biopsy specimens collected from each dog were fixed in 4% buffered paraformaldehyde solution for 15 min, rinsed in

phosphate solution (pH 7.4) for three days, put into 18% buffered sucrose solution and stored at 4 °C at least for three weeks. The fragments of the colonic mucosal layer were then frozen at –20 °C, cut to 10-µm sections with a Microm cryostat (HM525, Walldorf, Germany) and mounted on microscopic slides. The sections were examined by the single immunofluorescence technique according to Gonkowski et al. (2013). Briefly, the procedure was as follows: (a) drying for 45 min at room temperature (rt), (b) incubation with blocking solution (10% goat serum, 0.1% bovine serum albumin, 0.01% NaN_3, Triton X-100 and thiomersal in PBS) for 1 h to avoid non-specific binding of antibody, (c) incubation with anti-SP antibody (rat, Biogenesis Ltd, Poole, UK, catalogue no. 8450-0505, working dilution 1:500) in a humid chamber, overnight (rt), (d) incubation with a specific secondary antibody conjugated to Alexa Fluor 488 (donkey anti-rat, Abcam, Cambridge UK, catalogue no. ab150153, working dilution 1:1,000) for 1 h (rt), (e) covering with glycerol solution in PBS (1:2; pH 7.4) and coverslips. Among each of the above-mentioned labelling stages, the sections of the descending colon were rinsed in PBS (3 × 10 min).

Sections of the descending colon were studied using the Olympus BX51 fluorescence microscope equipped with appropriate filters. The number of intramucosal nerve fibres immunoreactive to SP was measured by a semi-quantitative method, which consisted of the counting of SP-LI fibres in the field of view (0.1 mm^2). The number of nerves was evaluated in four fields of view in three sections of every biopsy specimen. In every animal included in the study (control and suffering from IBD), a total of 36 fields of view were evaluated. The distance between the fields of view in which nerve fibres were counted was at least 100 µm to avoid repeated counts of the same structures. The results were grouped and the mean values and standard deviation were calculated. Microphotographs were captured with a digital camera in the Analysis 3.0 application. The specificity of the primary antibody used was verified by routine methods, including pre-absorption, replacement and omission tests, which completely eliminated specific staining.

The significance of differences between groups was determined by the Kruskal–Wallis test at $P \leq 0.05$ (significant) and $P \leq 0.01$ (highly significant). The results were processed in the Statistica 9.1 application (StatSoft, Inc.).

RESULTS

During the present investigation, intramucosal nerve fibres immunoreactive to SP were observed both in healthy dogs and in animals suffering from IBD at all severity stages (Figs 1 and 2). In the control animals, the average number of SP-LI nerve fibres was 10.199 ± 2.11 per observation field (Fig. 2A). In patients suffering from mild (Fig. 2B) and moderate (Fig. 2C) IBD, a slight increase in the number of nerves containing SP was noted, reaching 14.62 ± 2.86 and 14.80 ± 091 fibres per observation field, respectively (Fig. 1). However, the differences in the average number of nerves

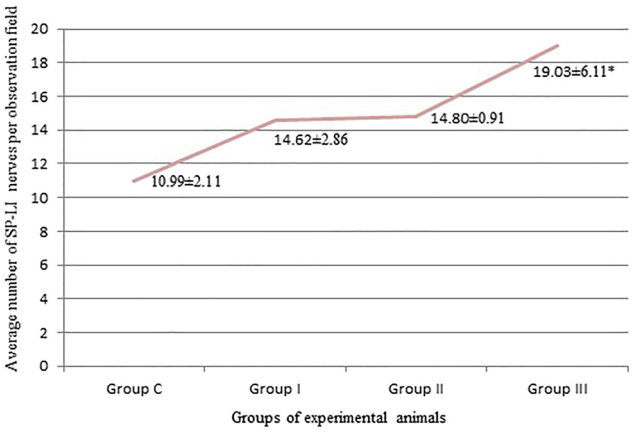

Fig. 1. Values are shown as the average number of substance P immunoreactive fibres ± SEM per field of view in the colon mucosa of healthy dogs and dogs with inflammatory bowel disease of varying severity: Group C – control group, Group I – animals with mild IBD, Group II – dogs with moderate IBD, Group III – dogs with severe IBD. *significantly different from the control

immunoreactive to SP between the control group and animals suffering from mild and moderate IBD were not statistically significant.

For dogs suffering from severe IBD, the average number of SP-LI nerve fibres was even higher and amounted to 19.03 ± 6.11 nerves per observation field (Fig. 2D). Contrary to patients with mild and moderate IBD, the differences between control animals and dogs with severe IBD were statistically significant ($P < 0.01$).

On the other hand, the influence of IBD on the appearance of SP-LI intramucosal colonic nerves was not observed during the present investigation. Both in control animals and in dogs suffering from IBD at every stage of severity, fibres immunoreactive to SP were similar. Most often, such fibres were relatively thin, short and delicate with visible varicosities (Fig. 2).

DISCUSSION

It is known that SP may play multiple roles within the GI tract, including the regulation of gut motility and GI tract secretion, participation in intestinal blood flow and the conduction of sensory stimuli, as well as having an impact on immunological reactions (Brunsson et al., 1995; Brehmer et al., 2004; De Fontgalland et al., 2008; Shimizu et al., 2008; Koon and Pothoulakis, 2006; Gross and Pothoulakis, 2007). The results obtained during the present study show that the activity of SP in intramucosal colonic nerves may be connected with the processes accompanying IBD in dogs. The relationships between IBD and the number of SP-LI nerves may result from two aspects. Although pathological changes during IBD may increase the number of SP-LI nerves in the mucosal layer in the canine descending colon, the elevated levels of SP caused by factors favouring IBD may be a predisposing factor for the disease symptoms. These observations made during the present study, together with the

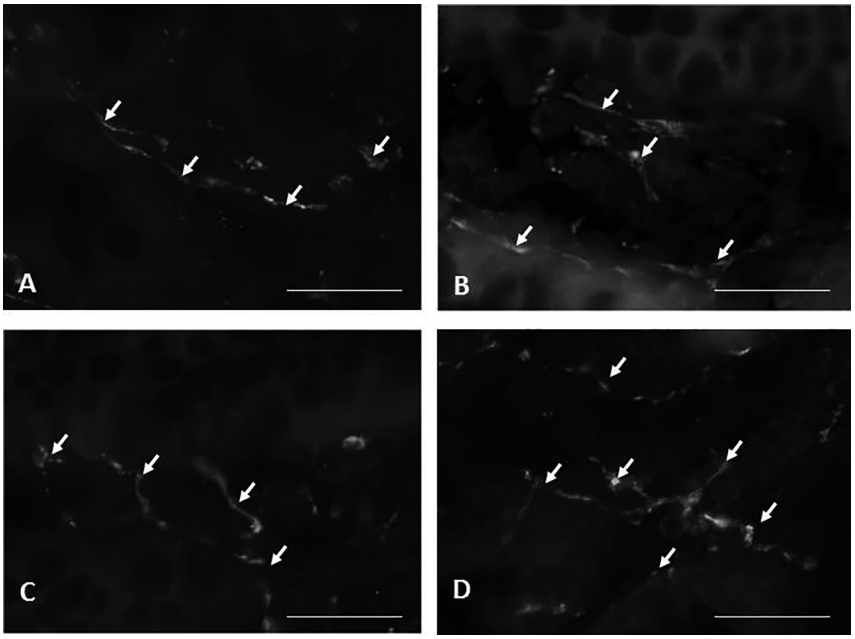

Fig. 2. Fluorescent microscope images showing intramucosal nerve fibres immunoreactive to substance P (indicated with arrows) in the descending colon of healthy dogs (A) as well as in dogs suffering from mild (B), moderate (C) and severe (D) inflammatory bowel disease. Scale bar: 40 μm

results of previous experiments where changes in the number of SP-positive of nervous structures supplying the GI tract were observed under the effect of various pathological and toxicological factors in other mammalian species (Vasina et al., 2006; Gonkowski, 2013; Makowska and Gonkowski, 2018; Szymanska et al., 2018), strongly suggest that SP takes part not only in the regulation of GI tract functions under physiological conditions but also in adaptive and/or protective reactions aimed at the maintenance of homeostasis.

It should be noted that knowledge about the influence of naturally occurring inflammatory processes in the GI tract on the expression of SP involved in intestinal innervation is rather scanty and primarily concerns ulcerative colitis and Crohn's disease in humans. During the first of the above-mentioned diseases, an increase in SP expression was observed in the large intestine, while Crohn's disease caused a decrease in the number of SP-positive nervous structures (Bernstein et al., 1993). To date, the influence of 'naturally' occurring intestinal inflammatory processes on SP-positive neuronal structures in the canine GI tract has not been studied, but the present results show that it is similar to that noted in humans.

It should be underlined that the exact mechanisms giving rise to the observed changes still remain unclear. They may be a result of the changes in various stages of SP synthesis and/or fluctuations concerning the transport of SP from cell body to nerve endings. Most likely, the changes induced by pathological stimuli in SP-positive nervous structures, noted both in the present study and in previous investigations, result from the relatively well-known participation of SP in immunological and inflammatory reactions (O'Connor et al., 2004; Koon and Pothoulakis, 2006; Gross and Pothoulakis, 2007). It is known that SP is one of the major proinflammatory factors which, by the activation of NK1 receptors located on the lymphocytes and macrophages, stimulate the production of potent proinflammatory cytokines, such as interleukin-1 and tumour necrosis factor alpha, among others (Mantyh et al., 1994). These cytokines modulate inflammatory processes and are connected with the clinical signs accompanying IBD, including vomiting and diarrhoea (Koon and Pothoulakis, 2006). The increase in the number of SP-positive nerves noted in the present study may also be associated with these signs. Previous studies have shown the close relationships of SP and its receptors with the appearance of vomiting (Okafor et al., 2017). Moreover, it is known that the intravenous administration of SP causes not only vomiting in dogs, but also increased faeces excretion, and the SP-antagonistic substances have antiemetic effects (Watson et al., 1995). Another key sign of canine IBD, potentially associated with an increase in the number of nerves immunoreactive to SP, is diarrhoea. Such associations have been described in humans, where SP induces histamine secretion by intestinal mast cells (Raithel et al., 1999) and chloride ion-dependent secretion in the colon during ulcerative colitis (Riegler et al., 1999). Moreover, it is known that SP may stimulate fibroblasts leading to fibrosis of the colonic mucosal layer characteristic of severe chronic colitis (Rieder and Fiocchi, 2008; Koon et al., 2010). The hypothesis that the last-mentioned activity of SP is behind the changes observed in the present study is all the more likely as the most pronounced changes have been noted in dogs suffering from severe IBD, where visible fibrosis was observed during histopathological examination. In view of the above-mentioned activities of SP, it cannot be excluded that it is not the processes connected

with IBD that cause the changes in the number of SP-LI nerves, but, on the contrary, the increase of SP levels in colonic innervation caused by factors inducing IBD leads to the changes specific for this disease.

Of course, it cannot be excluded that the increase in the number of SP-positive intestinal nervous structures in the canine GI tract during IBD may be connected with other mechanisms not directly related to the SP-induced activation of proinflammatory cytokines. One of these may be associated with the direct influence of SP on intestinal muscle contractility, which clearly depends on the animal species and the type of activated receptor (Lördal et al., 1993, 1997; Shimizu et al., 2008). It is probable that the increase in the number of SP-positive nerves noted in the present study is linked to the intestinal dysmotility occurring during IBD and resulting in diarrhoea, one of the clinical signs of this disease (Mawe et al., 2004).

Moreover, IBD-induced changes in the expression of SP within intestinal nervous structures may result from pain symptoms accompanying this disease, as well as fluctuations in the intestinal blood flow, leading to inflammatory hyperaemia of the intestinal mucosal layer, because SP is known to be an important neuronal factor participating in the conduction of sensory and pain stimuli (Brehmer et al., 2004; Rytel and Calka, 2016), as well as in the regulation of intestinal and mesenteric blood flow (Brunsson et al., 1995; De Fontgalland et al., 2008). On the other hand, the increase in the number of SP-positive intestinal nervous structures in dogs suffering from IBD could be a manifestation of defence reactions of the body against damaging factors occurring during disease, because it is known that SP is a factor that promotes intestinal regeneration, inhibits pathological changes in experimentally-induced inflammation of the GI tract and preserves the integrity of the intestinal mucosal layer (Hwang et al., 2017).

In conclusion, the results obtained during the present study clearly indicate that SP is a neuropeptide involved in processes connected with IBD in dogs, especially in the severe stage of this disease, during which a clear increase in the number of SP-positive nerves located in the mucosal layer of the canine colon has been observed. These changes are probably connected with the relatively well-known proinflammatory activity of SP, as well as with the adaptive and protective properties exhibited by this substance. However, the exact mechanisms underlying the observed changes are not fully clear and the explanation of all aspects requires further studies. Nevertheless, the present study may be the first step towards the eventual use of SP and its analogues in the treatment of canine IBD in the future, as well as towards incorporating the examination of the number of SP-positive intestinal nerve fibres into the diagnostic process.

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